Molecular analysis of accessory gene regulator functionality and virulence genes in *Staphylococcus aureus* derived from pediatric wound infections

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Abstract

Staphylococcus aureus is a major human pathogen causing infections with high morbidity and mortality in both healthcare and community settings. The accessory gene regulator (Agr) is a key genetic element controlling the expression of numerous virulence factors in *S. aureus*. The significance of a functional Agr system in clinical *S. aureus* isolates derived from pediatric wound infections is still unclear. Therefore, the present study was conducted to identify virulence genes and determine Agr functionality from this cohort of patients. A total of 48 *S. aureus* wound isolates were collected from patients referred to Tehran Children's Medical Center Hospital from April 2017 to April 2018. In addition, *in vitro* antimicrobial susceptibility of the isolates was assessed using the disk diffusion and E-test methods. Conventional PCR was performed for the detection of toxins (*tsst-1, hla, hlb, hld, eta, etb, etd, edin-A, edin-B, edin-C*) and Agr typing (*agrI, agrII, agrIII, agrIV*). Agr functionality was assessed by quantitative reverse transcriptase real-time PCR (qRT-PCR). All *S. aureus* isolates were found to be susceptible to linezolid and vancomycin. The most frequently detected toxin gene was *eta* (100%), and the most prevalent Agr type was *agrIII* (56.3%). Importantly, qRT-PCR revealed that Agr was functional in 28 (58%) of wound isolates. Consequently, our data suggests that a functional Agr system may not be required for the development of *S. aureus* wound infections.

Keywords: *Staphylococcus aureus*, Agr functionality, wound infections, pediatrics.

1. Introduction

Staphylococcus aureus is an ever-present opportunistic pathogen that can cause a variety of diseases. The severity of *S. aureus*-associated infections ranges from benign localized skin abscesses to life-threatening diseases, such as arthritis, osteomyelitis, and endocarditis (Francois *et al*., 2006; von Eiff *et al*., 2004). In recent decades, methicillin-resistant *S. aureus* (MRSA) strains have emerged as a predominant cause of invasive diseases, namely skin and soft tissues, as well as musculoskeletal infections in children (Kaushik and Kest, 2018). This bacterium is one of the most dominant commensals on human skin and nasal mucosa and can express a multitude of virulence factors, such as surface adhesins, enterotoxins and hemolysins which are central in the development of disease. (Kassam *et al*., 2017; Stevens *et al*., 2017). The synchronized expression of these virulence determinants is tightly controlled by the cumulative action of several regulatory elements, such as the accessory gene regulator (*agr*), staphylococcal accessory regulator A (*sarA*), and the alternative sigma factor B (σB) (Manna and Cheung, 2001).

The Agr system plays a central role in the growth-phase dependent modulation of virulence gene expression (Bronner *et al*., 2004; Sakoulas *et al*., 2003a). The *agr* operon is an autocatalytic system controlled in a cell density-dependent fashion through the production and sensing of auto-inducing peptides (AIP). At high cell density, the Agr system increases the production of many secreted virulence factors, including Toxic shock syndrome toxin -1 (TSST-1), delta-hemolysin and exfoliative toxins A and B (ETA and ETB). In contrast, Agr decreases the expression of several colonization factors such as fibronectin binding proteins, important in adhesion and biofilm formation (Li *et al*., 2018). The *agr* locus consists of two distinct transcripts, RNAII and RNAIII, which are transcribed by two promoters, P2 and P3 respectively. The activation of P2 induces the expression of the components involved in cell-to-cell quorum-sensing communication (AgrBDCA) (Bibalan *et al*., 2014a). Both AgrB and AgrD function to process and secrete the auto-inducing peptide (AIP), which acts as the chemical messenger critical for Agr activity (Wang *et al*., 2014). Upon reaching a critical density, AIPs interact with the sensor kinase, AgrC which promotes phosphorylation of the DNA binding response regulator AgrA. Phosphorylated AgrA undergoes a conformational change permitting interaction and binding to the intergenic region between P2 and P3 facilitating their expression. P3 activation leads to the expression of RNAIII, the effector of target gene regulation (Novick and Geisinger, 2008).

Several studies have demonstrated a correlation between *agr* types and particular diseases. For example phylogenetic group AF1 (*agr* group IV) strains are closely related to generalized exfoliative syndromes and bullous impetigo whereas endocarditis is mainly caused by phylogenetic group AF2 (*agr* groups II and I) strains (Jarraud *et al*., 2002). In addition, it has been suggested that *agr* group III and IV strains are associated with toxic shock syndrome (Gomes *et al*., 2005). To the best of our knowledge, there is no published study evaluating Agr functionality among Iranian *S. aureus* isolates. The present study was conducted to determine dominant Agr types, Agr activity and presence of specific virulence genes in *S. aureus* isolates derived from pediatric wound infections.

2.1 Materials and Methods

2.1 Bacterial isolation and identification

In the present study, 48 *S. aureus* isolates were collected from wound infections of pediatric patients referred to the Children's Medical Center Hospital Tehran, Iran over one year from April 2017-2018. The School of Medicine, Shahid Beheshti University of Medical Sciences ethics committee approved this study (IR.SBMU.MSP.REC.1395.369). The isolates were identified as *S. aureus* according to phenotypic (colonial morphology and Gram-stain), biochemical (catalase, haemolysis, oxidase, coagulase, DNase, and mannitol fermentation tests) and genetic (polymerase chain reaction (PCR) detection of *S. aureus* specific nuclease A (*nucA*) gene) analysis. The isolates were stored in Tryptic Soy Broth (TSB) (Merck, Germany) containing 20% glycerol at -80C for further investigation.

2.2 Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed using the Kirby-Bauer disc-diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (Huse *et al*., 2017). Commercially available antibiotic disks (Mast Co., UK) used in this study included penicillin (10 units), ciprofloxacin (5 μg), clindamycin (2 mg), gentamicin (10 μg), erythromycin (15 μg), linezolid (30 μg), oxacillin (30 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg) and ceftaroline (30 μg). In addition, susceptibility to vancomycin was determined using minimum inhibitory concentration (MIC) E-test strips (Liofilchem Co., Roseto, Italy*).*

2.3 MRSA identification

For detection of MRSA isolates, an MIC of cefoxitin was determined using E-test (Liofilchem Co., Italy). *S. aureus* ATCC 25923 was used as a quality control reference strain. Additionally, for the molecular detection of MRSA, PCR analysis of the *mecA* gene was performed (Table 1).

2.4 DNA extraction

Genomic DNA was isolated from bacterial strains grown in Mueller-Hinton broth (Merck Co., Germany) overnight at 37°C. Pelleted bacterial cells were resuspended in 200 µl of phosphate-buffered saline (PBS) and then DNA extraction was carried out using High Pure PCR Template Preparation Kit (Roche Co., Germany) according to the manufacturer's instructions. Evaluation of the concentration and purity of extracted DNA was measured by Nanodrop (DeNovix Inc., USA). Extracted DNA was stored at -20°C for later analysis.

2.5 Molecular detection of toxin genes and Agr typing

The presence of the virulence genes encoding toxins, (*tsst-1, hla, hlb, hld, eta, etb, etd, edin-A, edin-B, and edin-C*) were investigated using PCR. The product size and annealing temperature of each primer sets are provided in Table 1. Agr typing was conducted using a pan forward primer and four specific reverse primers (Table 1). The PCR reaction was performed in a total volume of 25 µl containing 12.5 µl of 2X master mix (BIOFACT, Korea), 1µl (10 pM/ µl) of each primer, 8.5 µl of distilled water, and 2µl of DNA (10 ng) template. The cycling programs, was preceded by 4 min at 94° C and consisted of 30 cycles of 94° C for 2 min, 1 min annealing at specific temperature for each primer set (Table 1) and 72° C for 1 min, followed by a final extension step at 72° C for 5 min. PCR amplicons were separate using 1.2% agarose gels and visualized by staining with gel red stain (CinnaGen Co., Iran).

2.6 Reverse transcription and quantitative RT-PCR

S. aureus isolates grown overnight in TSB were diluted 1:1000 in fresh TSB and grown at 37°C for 6 h. The cultures were treated with two volumes of RNAlater (Sigma-Aldrich, Germany), immediately mixed by gentle vortexing for 5 s and incubated for 10 min at room temperature. Next, cultures were centrifuged at 7000 × *g* for 10 min, supernatant discarded and the resulting pellet was stored at -70°C until required. Following thawing on ice, the pellet was resuspended in Tris-EDTA (TE) buffer (pH 8) containing 200 µg/mL lysozyme (Sigma-Aldrich Co., Germany)

and 250 µg/mL lysostaphin (Sigma-Aldrich Co., Germany) followed by incubation at 37°C for 2 h. During the incubation period, the suspension was mixed for 10s in 10 min intervals. Following incubation, the suspension was treated with proteinase K (Roche Co., Germany) for 20 min at 37°C with mixing for 10 s at 3 min intervals. RNA extraction was performed using the RNeasy Mini Kit (Roche Co., Germany) according to the manufacturer's instructions with the addition of an extra DNase treatment (CinnaGen Co., Iran) following RNA purification. The absence of DNA contamination was verified by PCR amplification of the housekeeping *gyrA* gene. Reverse transcription was carried out using the cDNA synthesis kit (Wizbio Co., South Korea) according to the manufacturer's instructions. Real-time PCR was performed with SYBR green PCR master mix (Amplicon Co., Denmark) using specific primers for both *gyrA* [*gyrA*F: 5'-CCAGGTAAATTAGCCGATTGC-3'; *gyrA*R: 5'-AAATCGCCTGCGTTCTAGAG-3'] and RNAIII [*rnaIII*F:5'-GAAGGAGTGATTTCAATGGCACAAG-3', *rnaIII*R: 5'-GAAAGTAATTAATTATTCATCTTATTTTTTAGTGAATTTG-3']. Cycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 20 s and 54°C for 1 min and a dissociation step 72°C for 20 s. The relative expression was normalized to the value of the positive control (*S. aureus* strain NCTC8325) as described previously (Gomes-Fernandes *et al*., 2017a). Accordingly, Agr functionality was determined as RNAIII expression of within 10-fold of the positive control as described previously (Gomes-Fernandes *et al*., 2017a). Experiments were performed using three biological replicates.

2.7 Statistical analysis

The data was analyzed with SPSS version 22.0 (IBM Corp., USA). Gene expression analysis was performed using REST® 2009 (Qiagen, Germany) software. Independent-samples t-test was used to evaluate differences between test groups. A *p-value* of less than 0.05 was considered statistically significant.

3. Results

3.1 Bacterial strains and antimicrobial resistance profiles

In this study, 48 *S. aureus* clinical isolates were collected from pediatric wound infections from children aged between 1 day and 14 years. 14 (29.2%) samples were collected from patients admitted to the in infectious disease ward, 9 (18.8%) from post-surgery ward, 15 (31.2%) from infants, 6 (12.5%) from OPD, 3 (6.3%) from in-patient ward, 2 (4.2%) from emergency cases, and 3 (6.3%) from gastrointestinal, 2 (4.2%) from neurosurgery, 2 (4.2%) from intensive care unit, 1 (2.1%) from coronary intensive care unit and1 (2.1%) from nephrology wards. The antibiotic susceptibility profile showed that all isolates were susceptible to linezolid and vancomycin. Clinical isolates were highly susceptible to ceftaroline (89.6%, n=43), trimethoprim-sulfamethoxazole (83.3%, n=40), and gentamicin (77.1%, n=37). Lastly, the susceptibility rate for ciprofloxacin, cefoxitin, erythromycin, and penicillin was determined 68.8% (n=33), 43.8% (n=21), 39.6% (n=19), and 2.1% (n=1), respectively (Table 2). The frequency of MRSA and MSSA were 57.6% and 43.7%, respectively based on both cefoxitin susceptibility and presence of *mecA* gene.

3.2 Distribution of toxin-encoding genes

We evaluated the prevalence of *S. aureus* toxin-encoding genes from strains isolated from pediatric wound infections using PCR and primers outlined in Table 1. Additionally, the resultant amplicons generated were sequenced and submitted to GENBANK, and the accession numbers for *tsst-1, eta, etb,* and *edin-C* genes are shown in Table 4. Our results showed that the *eta* was the most prevalent gene (100%), followed by *hld* (97.9%), *hla* (72.9%), *hlb* (60.4%), *edin-B* (47.9%), *tsst* (41.7%), *edin-C* (33.3%), *etd* (22.9%), *etb* (14.6%), *edin-A* (2.1%). Based on statistical analysis, there was no significant correlation between antibiotics resistance and toxins genes in MRSA and MSSA isolates (Table 3).

3.3 Agr typing

Agr type were determined by PCR using specific primers (Table 1). Generated PCR amplicons were sequenced and submitted to GENBANK with the accession numbers of *agrII, agrIII* shown in Table 4. The results indicate that *agrIII* gene (56.3%) was the predominant Agr type followed

by *agrI* (41.7%), *agrII* (8.3%), and *agrIV* (8.3%). Statistical analysis of virulence genes and Agr type indicated no significant association.

3.4 Agr functionality evaluation

In order to measure Agr activity, RNAIII expression was evaluated and compared with *S. aureus* strain NCTC8325 as a control. In total 48 wound isolates of *S. aureus* were assessed. Figure 1 illustrates the expression level of RNAIII among MRSA (Fig 1a) and MSSA (Fig 1b) isolates using qRT-PCR. Agr functionality was observed in 28 (58%) wound isolates with 56% of MRSA (15/27) and 61% of MSSA (13/21) being classed as Agr functional. We observed a significant correlation between the presence of *tsst-1* gene with Agr functionality (p=0.05). However no statistically significant association was observed between Agr functionality and the presence of toxin genes, methicillin resistance or Agr class type.

4. Discussion

The present study was conducted to evaluate the activity of the Agr system among clinical isolates of *S. aureus* derived from pediatric wound infections. In addition, the association between Agr activity and the presence of several virulence determinants and antibiotic susceptibility was examined. Several techniques can be used to determine Agr function including the CAMP synergistic haemolysis assay, the Vesicle Lysis Test (VLT) and qRT-PCR detection of RNAIII.

Agr activity is traditionally evaluated using the CAMP test, which reports on the expression of delta haemolysin, a 26-amino acid toxin translated from the *rnaIII* transcript (Novick and Geisinger, 2008). A previous study reported that the interpretation of the CAMP test for evaluation of Agr activity may be unreliable with results varying between different laboratories, particularly observed for *S. aureus* strains exhibiting weak haemolytic activity (Traber *et al*., 2008). Alternatively it is shown that the VLT method, a highly sensitive assay specific to toxins strictly regulated by Agr system, is more reliable than CAMP assay for Agr functionality assessment (Laabei *et al*., 2014). However, the evaluation of RNAIII expression using qRT-PCR is still considered the gold-standard for Agr activity assessment (Gomes-Fernandes *et al*., 2017b; Laabei *et al*., 2014). Accordingly, we investigated the expression of RNAIII as a marker for Agr activity using qRT-PCR in our cohort of *S aureus* strains.

Our results demonstrated that 58% of tested *S. aureus* isolates were Agr functional. The expression of virulence genes by *S. aureus* is influenced by the Agr system, which controls the balance of virulence factors known to be important during the colonization and invasive phases of infection (Papakyriacou *et al*., 2000). To the best of our knowledge, there are no published studies examining Agr functionality among clinical isolates of *S. aureus* derived from pediatric wound infections. A recent study reported that Agr activity was high (82.2%) in *S. aureus* strains isolated from lower respiratory tract infections (Gomes-Fernandes *et al*., 2017b). These findings suggest the importance of a functional Agr system in lower respiratory tract colonization and infection. In contrast, previous work has highlighted that genes encoding the Agr system are down-regulated in cases of persistent bacteremia (Malachowa *et al*., 2011). However, a recent review of numerous studies highlighted that the percentage of Agr dysfunctional strains isolated from bacteraemia varies widely (3-82%) (Painter *et al*., 2014). This most likely reflects the different methods in testing Agr and different genetic backgrounds of *S aureus* strains. Different infections may promote the emergence of Agr dysfunction. It has also been demonstrated that apolipoproteins in human blood can interfere and inhibit Agr activity (Reuter *et al*., 2016) whereas this selection for downregulating Agr function may not be as strong in other infections.

The Agr typing results revealed that the majority of isolates belonged to Agr Group III followed by Agr Group I, Agr Group II, and Agr Group IV. In accordance with our data, two recent studies highlighted that Agr Group III was the predominate Agr group derived from hospital clinical isolates (Bibalan *et al*., 2014b) (Ben Ayed *et al*., 2006). The exact relationship between specific Agr groups and particular infections is not clear, however past studies have highlighted significant associations between the two factors. For instance, past work reported that the majority of menstrual toxic shock strains belonged to Agr specificity Group III and exfoliative toxin producers responsible for staphylococcal scalded skin syndrome (SSSS) and bullous impetigo were designated Agr Group IV (Jarraud *et al*., 2000). Furthermore, it was observed that TSST-1 producing strains belonged to *agr* Groups I and III (Chini *et al*., 2006). Additionally, isolates taken from patients suffering from endocarditis were mainly associated with with *agr* Group I (Gomes *et al*., 2005). Finally in a study investigating Agr activity in bloodstream isolates it was reported that more than half of strains belonged to agr group II (Sakoulas *et al*., 2003b). Our analysis

showed that there was no significant correlation between Agr types and *S. aureus* isolates from wound infection however the majority of isolates belonged to Agr group III.

Previous studies have focused on examining the relationship between Agr functionality and susceptibility to some antimicrobial agents. The most prominent observations highlighted a reduction in vancomycin susceptibility in Agr dysfunctional isolates (Soon *et al*., 2017; Tsuji *et al*., 2012; Tsuji *et al*., 2007). In this study, we observed no association between antibiotic resistance and Agr dysfunction. One limitation of this study was the relatively low sample size of isolates tested. Additionally, we did not screen for nasal carriage nor genotype the .*S aureus* isolates. These additionally tests would indicate whether the infecting *S. aureus* isolate was part of the patient's microflora or had been introduced externally from the hospital environment. Future studies will incorporate these analyses and improve our understating of *S. aureus* wound infections.

5. Conclusion

In the present study, the data revealed that there was no significant correlation between Agr activity and the ability to cause wound infections by *S. aureus* strains.

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Table 1. Oligonucleotide primers used in this study.

Target	Sequences(5'-3')	Annealing temperature (C°)	Product size (bp)	Reference
nuc	F: GCGATTGATGGTGATACGGTT	54	270	(Stuhlmeier and Stuhlmeier, 2003)
	R: AGCCAAGCCTTGACGAACTAAAGC			
mecA	F: GTAGAAATGACTGAACGTCCGATAA	60	310	(Seni *et al*., 2013)
	R CCAATTCCACATTGTTTCGCTCTAA			
tsst-1	F: TTATCGTAAGCCCTTTTGTTG	46	398	(Benvidi *et al*., 2017)
	R: TAAAGGTAGTTCTATTGGAGTAGG			
hla	F: CTGATTACTATCCAAGAAATTCGATTG	53	210	(Suryadevara *et al*., 2012)
	R: CTTTCCAGCCTACTTTTTTATCAGT			
hlb	F: GTGCACTTACTGACAATAGTGC	53	310	(Suryadevara *et al*., 2012)
	R: GTTGATGAGTAGCTACCTTCAGT			
Hld	F: GAATTTGTTCACTGTGTCG	49	357	(Kiran *et al*., 2009)
	R: TTTACACCACTCTCCTCAC			
eta	F: TTTGCTTTCTTGATTTGGATTC	51	464	(Koosha *et al*., 2014)
	R: GATGTGTTCGGTTTGATTGAC			
etb	F: ACGGCTATATACATTCAATT	51	226	(Suryadevara *et al*., 2012)
	R: TCCATCGATAATATACCTAA			
etd	F:GGGGAGACTATAGCTTCTGGTGTATTA	55.5	477	(Franke *et al*., 2010)
	R: TCCAACATGAATACCAACTAACTCT			
edinA	F: TAAATGGGGGAATAAACTTA	43	248	(Yamashita *et al*., 2013)
	R: CGATACTTGTCAAATAATCT			
e*dinB*	F: CATAAATACTCCTCTAAG	40	444	(Ohkura *et al*., 2009)
	R: GCATATTCTGTCCCTCTA			
edinC	F: TATTAAGCATTCATTCAA	45	629	(Ohkura *et al*., 2009)
	R: AGTGTAGTCTGTTCCTCT			
agr	Pan F: ATGCACATGGTGCACATGC		-	
	R1: GTCACAAGTACTATAAGCTGCGAT	54.5	439	(Suryadevara *et al*., 2012)
	R2: TATTACTAATTGAAAAGTGCCATAGC	54	573	
	R3: GTAATGTAATAGCTTGTATAATAATACCCAG	54.5	406	
	R4: CGTAATGCCGTAATACCCG	56	657	

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Table 2. The antimicrobial susceptibility patterns of *S. aureus* isolated from wound infection of pediatric patients

Antibiotics	(%) Resistance	(%)Intermediate	(%) Susceptible
Penicillin	(97.9%)	0	(2.1%)
Erythromycin	(45.8%)	(14.6%)	(39.6%)
Clindamycin	(41.7%)	(12.5%)	(45.8%)
Cefoxitin	(56.3%)	0	(43.8%)
trimethoprim-sulfamethoxazole	(16.7%)	0	(83.3%)
Oxacillin	(54.2%)	0	(45.8%)
Linezolid	0	0	(100%)
gentamicin	(22.9%)	0	(77.1%)
ceftaroline	(8.3%)	(2.1%)	(89.6%)
ciprofloxacin	(27.1%)	(3.4%)	(69.5%)
vancomycin	0	0	(100%)

Table 3. The frequency of the virulence and Agr genes determinant among the MRSA and MSSA isolated in *S. aureus*

gene	MRSA	MSSA	P value *
tsst-1	37.28%	**38.09%**	0.771
hla	66.6%	**80.9%**	0.338
hlb	66.6%	**52.38%**	0.380
hld	100%	**95.23%**	0.438
eta	100%	**100%**	1
etb	7%	**23.8%**	0.118
etd	25.92%	**19.04%**	0.733
edinA	3.7%	**0%**	1
edinB	40.7%	**57.14%**	0.383
edinC	25.92%	**42.85%**	0.237
agrI	40.74%	**42.85%**	1
agrII	7.4%	**9.5%**	1
agrIII	66.6%	**42.85%**	0.144
agrIV	7.4%	**9.5%**	1

* p values were measured using a Chi-squared analysis

Table 4. Accession numbers of genes

Target gene	Accession Numbers
tsst-1	MH805860
eta,	MH727607
etb	MH818223
edin-C	MH750913
agrII	MH805858
agrIII	MH805859

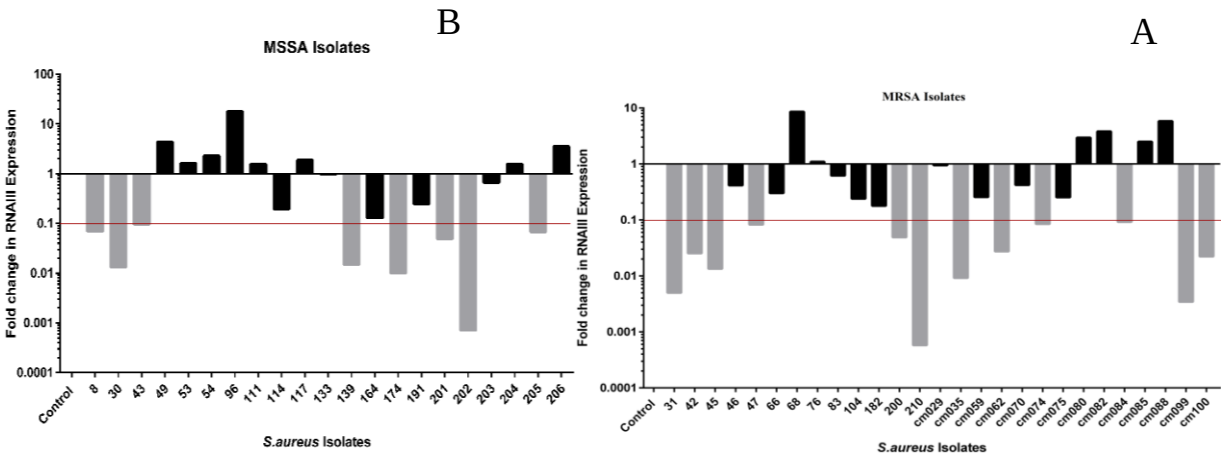

Figure 1. The expression level of RNAIII among MRSA (A) and MSSA (B) strains.
27 MRSA (A) and 21 MSSA (B) isolates were analyzed for Agr activity using qRT-PCR. Fold change of RNAIII expression was normalized to the housekeeping gene *gyrB*. The red line depicts the cut off for functional Agr activity based on a 10-fold difference to a positive Agr control.